

**Title:**

**Neutral variation does not predict immunogenetic variation in the European grayling (*Thymallus thymallus*) – implications for management**

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**Running title:** Neutral and functional variation in grayling

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## 28 Abstract

29 Preservation of genetic diversity is critical to successful conservation and there is  
30 increasing demand for the inclusion of ecologically meaningful genetic information in  
31 management decisions. Supportive breeding programmes are increasingly  
32 implemented to combat declines in many species, yet their effect on adaptive genetic  
33 variation is understudied. This is despite the fact that supportive breeding may  
34 interfere with natural evolutionary processes. Here, we assessed the performance of  
35 neutral and adaptive markers (Major Histocompatibility Complex; MHC) to inform  
36 management of European grayling (*Thymallus thymallus*), which routinely involves  
37 supplementation of natural populations with hatchery-reared fish (stocking). This  
38 study is the first to characterize MH II DAA and DAB loci in grayling and to  
39 investigate immune genetic variation in relation to management practice in this  
40 species. High-throughput Illumina sequencing of 'introduced', 'stocked native' and  
41 'non-stocked native' populations revealed significantly higher levels of allelic richness  
42 and heterozygosity for MH markers than microsatellites exclusively in non-stocked  
43 native populations. Likewise, significantly lower differentiation at the MH II than for  
44 microsatellites was apparent when considering non-stocked native populations, but  
45 not stocked populations. We developed a simulation model to test the effects of  
46 relaxation of selection during the early life stage within captivity. Dependent on the  
47 census population size and stocking intensity, there may be long-term effects of  
48 stocking on MH II, but not neutral genetic diversity. This is consistent with our  
49 empirical results. This study highlights the necessity for considering adaptive genetic

variation in conservation decisions and raises concerns about the efficiency of stocking as a management practice.

## Introduction

In order to manage biodiversity in the light of elevated rates of species extinctions (Ceballos et al. 2015), it is acknowledged that the consideration of genetic variation is crucial (Pertoldi et al., 2007; Sgrò et al., 2011). In the short-term, the loss of genetic variation directly impacts population viability due to negative effects associated with inbreeding depression (Spielman et al., 2004). In the long-term, populations are expected to persist in a changing environment only if they harbour sufficient adaptive potential (Duploux et al., 2013). The management of adaptive genetic variation is therefore at the core of conservation genetics (Allendorf et al., 2010). Indeed, there is great promise in measuring adaptive genetic variation because it makes the consideration of evolutionary dynamics possible, which may greatly improve the effectiveness of conservation planning (Brodersen and Seehausen, 2014). Assessing adaptive genetic variation directly is important because neutral variation may be affected differently by demographic processes (e.g. through bottlenecks (Ejsmond and Radwan, 2011; Sutton et al., 2011)), thus conservation decisions based solely on assessment of neutral variation may be poorly informed. Whilst neutral marker surveys continue to be in wide use in conservation genetics due to their convenience, repeatability and low cost, increasingly, there are calls to study the dynamics of functional genetic variation underlying ecologically meaningful traits in conservation genetic studies (Piertney and Webster, 2010). Despite the great promise in monitoring and managing adaptive

genetic variation, it has only become feasible to do so at a large scale in recent years due to the increasing accessibility of whole genomic screening techniques (Koboldt et al., 2013).

The assessment of adaptive genetic variation is particularly important where species management includes captive breeding. Although captive breeding is an important management tool to reduce biodiversity loss (Frankham, 2008; Griffiths and Pavajeau, 2008), it can interfere with adaptive processes (Ayllon et al., 2006). A good example is supportive breeding where adults are maintained temporarily in captivity to produce offspring that are released into the wild population. Neff et al. (2011) found that evidence for successful restoration of stable populations through supportive breeding is rare. Failing to preserve adaptive genetic variation is potentially one of the main causes of the ineffectiveness of current supportive breeding programmes, but more evidence is required to assess this (Neff et al., 2011). Supportive breeding is predicted to affect both neutral and adaptive genetic diversity in some contexts (like the reduction of the effective population size through unequal reproductive contributions of hatchery fish (Ryman and Laikre, 1991)), but in others may only affect adaptive and not neutral variation. For example, both the lack of natural selection acting on early life stages (Lynch and O'Hely, 2001) (which might be particularly important in species with high rates of juvenile mortality (de Eyto et al., 2011)), and the lack of natural mate choice in supportive breeding programmes can interfere with the preservation of adaptive genetic variation (Quader, 2005). It is therefore crucial to enhance our understanding of the effects of supportive breeding on adaptive genetic variation for this management technique to become a more fruitful conservation tool.

An important adaptive marker in the context of optimizing fitness of offspring from artificial breeding programmes is the immune related Major Histocompatibility Complex (MHC)(Hedrick, 2003; Pitcher & Neff, 2007; Ujvari & Belov, 2011). Protein products of the MHC have a central role in the recognition and elimination of foreign peptides and pathogens (Zinkernagel and Doherty, 1974). A large body of evidence demonstrates an association between MHC variation or specific MHC variants with overall or pathogen specific resistance (e.g. Evans and Neff, 2009; Meyer-Lucht and Sommer, 2005; Miller et al., 2004; Savage and Zamudio, 2011). Pathogen-mediated selection through negative frequency dependent selection and heterozygote advantage are thought to be the main mechanisms maintaining high diversity in the MHC and can lead to habitat specific MHC gene diversity (Eizaguirre, Lenz, Kalbe, & Milinski, 2012). Sexual selection has also been implicated in maintaining polymorphism through MHC-mediated mate choice in a range of taxa (Consuegra and Leaniz, 2008; Setchell et al., 2010; Strandh et al., 2012). Whilst the MHC does not represent overall adaptive variation, the loss of variation at this marker can have a strong negative effect on fitness, e.g. in inbred populations (Arkush et al., 2002), and standing genetic variation at the MHC is particularly important in the context of developing resistance to emerging disease (Dionne et al., 2009). The MHC is therefore widely recognized as a key marker for monitoring adaptive genetic variation in a conservation context (Eyto et al., 2007; Sommer, 2005; Ujvari and Belov, 2011).

Supportive breeding is becoming a widespread tool to re-invigorate species of conservation concern (Manlick et al., 2017; Moorkens, 2018; Tapley et al., 2015). It is a particularly common management strategy in salmonids (Fraser, 2008), so they are a good model to investigate its effectiveness in meeting conservation goals. European grayling (*Thymallus thymallus*) is a non-anadromous salmonid fish species with a wide distribution, ranging from France and Great Britain in the West to the Ural mountains in the East and from Montenegro in the South to Scandinavia in the North (Gum et al., 2009). The species is listed as protected in Appendix II of the Bern convention (Swatdipong et al., 2010) and UK populations are considered endangered (Dawnay et al., 2011). A number of pathogens and parasites are known to infect grayling (Dorovskikh and Stepanov, 2009; Pylkkö et al., 2006), likely imposing selection pressures on natural populations. There are also emerging threats to grayling such as proliferative kidney disease (Wahli et al., 2002) and the spread of disease from fish farms (Algöet et al., 2009). European grayling exhibit a high degree of spatial genetic structure across their natural range in the UK and continental Europe (Dawnay et al., 2011; Koskinen et al., 2002). In the UK, limited gene flow was detected between populations and four demographic clusters have been identified (Dawnay et al. 2011). To compensate for declines, supportive breeding (stocking) is a common practice to manage natural populations (Dawnay et al., 2011; Persat et al., 2016) and stocking policy in the UK has been altered in response to neutral genetic data in order to avoid homogenisation of genetically differentiated populations (Environmental Agency, 2011; Dawnay et al., 2011). However, so far only neutral genetic markers have been assessed and there is no information available on adaptive genetic variation.

Here, we combine empirical and modelling approaches to examine functional MHC genetic diversity of the class II  $\alpha$ -chain (DAA) and  $\beta$ -chain (DAB) in European grayling and its relationship with neutral genetic diversity. Specifically, we test (i) the degree to which neutral genetic variation reflects MHC genetic variation; (ii) the degree to which risk assessment of population viability and the definition of management units based on neutral genetic variation are consistent with results from MHC genetic variation; (iii) if there is an effect of management on neutral and adaptive genetic variation; (iv) using forward simulations we explore whether these effects can be explained by the lack of natural selection acting on hatchery produced offspring during captivity.

## Materials and Methods

### *Tissue Samples*

Thirty-seven to forty individuals from each of twelve populations were used for this study. These samples are a subset of those previously genotyped at ten microsatellite loci by Dawnay et al. (2011, see Figure 1). Five of the sampled populations are classified as ‘non-stocked native’ (Dee, Severn, Ure, Wye and Wylfe), four as ‘stocked native’ (Aire, Derbyshire Derwent, Dove and the Hampshire Avon) and three as introduced populations (Clyde, Eden and Itchen) (Dawnay et al., 2011). These populations represent all four demographic units (DUs) that were identified by Dawnay et al. (2011). The introduced populations are thought to be sourced from the Dove and Derbyshire Derwent, with one or more introductions taking place over the past 200 years (Ibbotson et al., 2001; Wilson, 1963). For the stocked native populations detailed information available on exact timing and

numbers of stocking events is limited. In the River Aire stocking was performed every year between 2006-2009, releasing between 1000 and 2000 individuals (Environmental Agency UK, personal communication). In the River Dove 1500 individuals were stocked in 2007 (Environmental Agency UK, personal communication). The age of stocked fish was between ~6-18 months (0+ or 1+). The likely provenance of the stocked fish is the river Test (Dawnay et al., 2011).

#### *MH II target loci*

In teleost fish, class I and class II major histocompatibility genes are not within one complex like in other vertebrates and hence are designated as MH (Stet et al., 2003). Our methods target variation at the MH class II  $\alpha$ -chain (DAA) and  $\beta$ -chain (DAB), covering most of the class II peptide binding region (PBR). Primer sequences for the DAA exon were based on published primers developed for brown trout (*Salmo trutta*; Stet et al., 2002; amplicon length: ~213 bp). Previously described primers for the  $\beta$ 1 domain encoded by exon 2 of the DBB gene, involved in peptide binding, were modified from those described by Pavey et al. (2011) (forward: 5'-ATGTTTTTCCTTTTAGATGGATATTTT -3', reverse: 5'-GTCTTATCCAGTACGACAC -3'; amplicon length: ~286 bp).

#### *NGS library preparation*

Tagged sequencing was used in a nested PCR, with the outer primer containing the Illumina adapter sequences and tags and the inner primer the target-specific sequence (after Lange et al., 2014). This allows different inner primers to be used



with the same set of tagged outer primers and is therefore flexible and cost-efficient. The assay was designed as a one-step PCR on a Fluidigm Access Array microfluidic chip (Lange et al., 2014), but was modified here to a two-step PCR for conventional thermocyclers.

Library preparation using PCR was performed as follows. Inner target-specific PCR was performed with a total volume of 6  $\mu$ l, containing 3.75 mM  $MgCl_2$ , 0.2 mM each dNTP, 4% DMSO, 0.2  $\mu$ M of each target-specific primer, FastStart High Fidelity Reaction Buffer and 0.15 U of FastStart High Fidelity Blend Enzyme (Roche/Sigma Aldrich) on Prime (Bibby) PCR cyclers or in a ABI 1 PCR cycler. Amplification used a thermal profile of: 95°C for 10 min, followed by 15 cycles at 95°C for 25 s, target-specific temperature and annealing time and 72°C for 90 s, and a final extension at 72°C for 5 min. Target-specific temperatures and annealing times were 59°C for 60 s (DAA locus) or 60°C for 45 s (DAB). PCR products were diluted (1:20) in  $H_2O$  and 3  $\mu$ l used as template in the second PCR which was carried out in a total volume of 7  $\mu$ l containing 3.75 mM  $MgCl_2$ , 0.2 mM each dNTP, 4% DMSO, 0.1  $\mu$ M of each outer primer, FastStart High Fidelity Reaction Buffer and 0.25 U of FastStart High Fidelity Blend Enzyme (Roche/Sigma Aldrich). The thermal profile of the second PCR was 95°C for 10 min, followed by 27 cycles at 95°C for 25 s, 60°C for 60 s and 72°C for 90 s, and a finishing step at 72°C for 5 min. Amplification success was verified on 20% of samples using 1% agarose gels. All samples were prepared in independent replicates along with ten randomly distributed negative controls for each locus, which represent samples without DNA input. Subsequently, PCR products were pooled per locus for each population prior to purification using AmpureXP (Beckmann and Coulter) and quantification using a Qubit 3.0 Fluorometer (Thermo Fisher Scientific). All populations were then pooled for each locus (equimolar concentrations) and run

on an Agilent 2100 Bioanalyzer to check product size and successful removal of unincorporated adapters and primers. Samples were then pooled in equal concentrations across loci and sequenced using an Illumina Miseq Nano (250bp paired end).

#### *Data analysis*

For quality control, all reads with a quality score below 20 in more than 90 percent of the sequence were filtered using the Filter by Quality tool on Galaxy Server (Goecks et al., 2010). Only sequences with both paired-end reads of sufficient quality were retained and aligned to each other using Mothur (Schloss et al., 2009). Primer mismatches (>1bp) and frame-shifts were filtered and examined for repeated sequences that could be derived from co-amplifying pseudo-genes. Read counts were adjusted if a variant (unique sequence) was present in a negative control. In this case, the highest read count of the variant observed in a control was subtracted from all amplicons where this variant was detected. Genotyping was performed if amplicons had a minimum of twenty reads. Because of the challenges associated with genotyping highly variable multi-gene families such as the MHC (Lighten et al., 2014a), like the distinction between natural recombinants and artificial chimeras, our genotyping approach builds upon the combination of two previously described pipelines to allow for high genotyping confidence. Briefly, genotyping was primarily done following the method described by Sommer et al. (2013), which is based on the comparison between replicate samples and also accounts for differences in allele amplification efficiency. Where the most frequent variant within one amplicon was not present within the technical replicate, an assignment error was assumed and the

individual excluded from the analysis. Where no replicate sample was available the genotyping methods described by Lighten et al. (2014b) were used as an additional criterion to assure genotyping confidence. Non-replicated genotyping estimates were only considered if they were consistent between the ‘Sommer’ and ‘Lighten’ estimate. The methods described by Lighten et al. (2014b) are based on the calculation of the degree of change (DOC-method) between variants and the comparison of read numbers to expectations under a number of alternative copy number scenarios (CNV method). The CNV-method was also applied to compare the effect of control read subtraction on the overall fit of the data to specific copy number scenarios. In this case up to five loci were considered. Here, the F-ratio test was used to decide whether control read subtraction resulted in significantly lower variance and better fit.

### *Genetic diversity analysis*

Summary statistics of genetic diversity were calculated for all populations. Conformity to Hardy-Weinberg equilibrium and allele frequency difference amongst populations were investigated using the Fisher’s exact test implemented in Genepop (Rousset, 2008) using 10,000 dememorizations, 100 batches and 10,000 iterations per batch. Observed and expected heterozygosity were calculated in GenAlex 6.5 (Peakall and Smouse, 2012). F-statistics (Weir and Cockerham, 1984) and allelic richness were calculated using Fstat (Goudet, 2001). Whilst Weir and Cockerham’s  $F_{ST}$  can be biased by differences in mutation rates (Hedrick 1999), which can be elevated for microsatellite markers, here variation at microsatellites was not higher than for MH markers, so that  $F_{ST}$  was considered sufficient to reflect differentiation

(Whitlock, 2011). Tests of significant differences of  $F_{IS}$  estimates and of  $F_{ST}$  estimates were based on 24000 randomisations and 66000 permutations respectively. The peptide binding region (PBR) was inferred by alignments of grayling MH II sequences to human HLA sequences (Brown et al., 1993). Amino acid (AA) diversity was calculated for the PBR as p-distance within and across populations in MEGA 7.021 and also as average pairwise p-distance across individuals for the whole sequence and only for the PBR (Kumar et al., 2016). A Mann-Whitney-Wilcoxon test was used to compare the pairwise p-distance across individuals for the whole sequence and only for the PBR.

MH locus data were compared to neutral microsatellite diversity for the same populations to evaluate how well neutral genetic variation reflects ecologically meaningful genetic variation. We conducted a simulation analysis by sequentially removing two microsatellite loci and measuring their correlation with the remaining eight loci over 1000 bootstrap cycles for all standard measures of genetic diversity to assess our ability to detect significance.

To detect differences in functional relative to neutral genetic variation across management classes, differences in observed and expected heterozygosity, inbreeding coefficient  $F_{IS}$  and allelic richness between MH II and microsatellites were tested for non-stocked native, stocked native and introduced populations. This was done using a clustered Mann-Whitney-Wilcoxon test implemented in the R-package 'clusrank', using the Datta-Satten method and 1000 bootstrap cycles (Jiang et al., 2017), to account for the dependency of measurements derived from the linked DAA and DAB genes respectively. The Kruskal-Wallis test was used to identify differences in measurements of genetic diversity across management classes for each marker type. Pairwise  $F_{ST}$  estimates were compared using a Mann-Whitney-Wilcoxon test

between MH II and microsatellite loci, using (i) all populations, (ii) non-stocked native and stocked native or (iii) only non-stocked native populations. For all tests involving multiple comparisons, the Benjamini-Hochberg method was used to correct for multiple testing (Hochberg and Benjamini, 1990). To assess whether population structure reflected by neutral markers is supported by adaptive genetic differentiation, a neighbour-joining phylogenetic tree was built based on Nei's genetic distance (Nei, 1972) in PHYLIP using a consensus of 2000 bootstrapped replications for all genes studied (Felsenstein, J, 1989). An analysis of molecular variance was done for both microsatellite and MH II data in GenAlex 6.5 (Peakall and Smouse, 2012).

### *Inference of selection*

Recent effects of selection on each gene and population were evaluated in ARLEQUIN 3.5 (Excoffier and Lischer, 2010) using a Ewens-Watterson homozygosity test (Ewens, 1972; Watterson, 1978). The Ewens-Watterson test compares allele frequencies observed within each population to those expected under neutrality for populations at mutation-drift equilibrium. The test assumes population equilibrium and is sensitive to demographic changes. During population bottlenecks low frequency alleles are lost at a higher rate, producing allele frequencies that are more even than expected under neutrality (Ewens, 1972; Watterson, 1978). Similarly population expansion leads to an increase in low frequency alleles and lower heterozygosity than expected under neutral-equilibrium (Meyer et al., 2006). In order to distinguish demographic and selective forces and their effect on allele frequency changes a Ewens-Watterson test was also performed

on the microsatellite data from Dawnay et al. (2011) for all populations studied. Where recent demographic events are the reason for deviations from neutrality both neutral and adaptive markers are expected to be affected, whilst selection is expected to only affect MH II markers. Following Larson et al. (2014) alpha margins of 10% ( $p < 0.1$ ,  $p > 0.9$ ) were considered as evidence of selection, because of the limited statistical power of the Ewens-Watterson test in detecting weak or moderate selection (Ewens, 1972) .

### *Simulations*

We implemented a simulation model using simuPop, version 1.1.8.3 (Peng and Kimmel, 2005), in order to investigate whether the lack of natural selection during early life-stages of hatchery reared juveniles could result in changes in observed and expected heterozygosity in supplemented populations where population census size differs (script available on request). We assumed a natural population with constant size and with an age class structure as described for grayling populations in Woolland and Jones (1975). We assumed age-dependent female fecundity (Charles et al., 2006). We assumed allele frequencies for DAA and DAB MH loci were the same as our estimates of the native Dee population (this study). We used a heterozygote advantage model for offspring survival with a selection coefficient of 0.1, which is within the range reported for loci under balancing selection in natural populations (0.05-0.15, Aguilar et al., 2004) and a model without selection to represent a comparable neutral marker reference. Thus, the probability of survival was given by the average fitness value across the two MH loci divided by the sum of probabilities across all individuals of a certain age class. We did not evaluate the

scenario of using foreign stocks and introducing potentially maladaptive alleles as this does not represent the generally recommended practice in a conservation context and is not the current practice of the Environmental Agency for grayling in the UK (Dawnay et al., 2011). After simulating the evolution of the population for ten years, ten adult males and females were selected randomly to produce the simulated hatchery offspring, before the adults were returned to the population. Selection-dependent survival on hatchery produced offspring was removed in the first year, before 1000 individuals at the age of 1 year were stocked into the source population. Of these individuals 50% were randomly removed from this cohort to simulate non-genetic effects of high initial mortality in stocked fish (Pedersen et al., 2003). This stocking procedure was simulated in three consecutive years and the allele frequencies in the population monitored for another ten years. Stocking intensity and frequency generally followed those actually practiced in the native stocked populations described above (Environmental Agency UK, personal communication). Stocking intensity was kept constant for different source population sizes of 500, 750, 1500 and 2000, so that the ratio of naturally produced offspring surviving to an age of one year to those stocked that initially survived (50%) were roughly 0.5:1, 0.8:1, 1.6:1 and 2:1 respectively. The population was replicated with or without stocking 100 times respectively. Observed and expected heterozygosity was compared between them, across the following ten years after stocking, using a Mann-Whitney-Wilcoxon test. Differences between stocked and non-stocked replicates were also tested for significance in each year after stocking for neutral and MH markers using a Mann-Whitney-Wilcoxon test.

## 364 Results

### 365 *Quality control*

366 The Illumina Nano run resulted in  $n=1,227,780$  reads. A small number of reads were  
367 observed in negative controls: the mean reads for these across 10 control samples  
368 respectively were  $10 \pm 26$  (total 103) and  $1.8 \pm 6$  (total 19), representing 0.02% and  
369 0.005% of the total reads for the DAA and DAB locus respectively. The fit of the  
370 overall dataset to specific copy number scenarios was significantly better after  
371 control read subtraction (F-test:  $F=0.67$ ,  $p<.0005$ ). Genotypes were obtained for a  
372 total of 389 individuals for the DAA and 359 individuals for the DAB locus. Of these  
373 82% were derived from replicated samples for the DAA and 52% for the DAB locus.  
374 Several samples were excluded from the analysis due to a potential assignment  
375 error (DAA  $n=7$ ; DAB  $n=1$ ), where the most frequent variant within one amplicon was  
376 not present in the replicate. The genotypes of most individuals were consistent with  
377 the single classical class II locus system found within other salmonids (Stet et al.,  
378 2002). However, individuals for the DAA ( $n=4$ ; 1%) and DAB ( $n=1$ ; 0.3%) loci  
379 exhibited three alleles and were excluded from subsequent analysis. The mean per  
380 amplicon coverage was 143 for the DAA locus and 89 for the DAB locus. For the  
381 DAA and DAB locus 15 and 10 alleles were identified, of which 14 and 10 encoded  
382 different protein sequences respectively.

383

### 384 *Genetic diversity*

385 The total number of alleles per population ranged from 2-7 for the DAA locus and 2-6  
386 for the DAB locus. Two populations, the stocked native Aire (AIR) and non-stocked



native Severn (SEV), showed significant heterozygote deficits (Table 1) and departure from HWE for the DAB gene.  $F_{IS}$  estimates ranged from -0.22 to 0.33 for the DAA locus and from -0.2 to 0.6 for the DAB locus. Average AA diversity within the PBR was 0.11 for DAA and 0.41 for DAB across all populations. There was greater within-population than between-population AA diversity for all but the Eden population for the DAB locus (Table 1). Average pairwise AA distance across individuals was significantly higher for the PBR than for the whole sequence for both the DAA and DAB locus (Mann-Whitney-Wilcoxon test:  $p < 0.001$ ).

No significant correlations were observed between microsatellite and MH expected heterozygosity (Spearman:  $\rho = 0.26$ ,  $p = 0.22$ ), observed heterozygosity (Spearman:  $\rho = 0.39$ ,  $p = 0.06$ ), allelic richness (Spearman:  $\rho = 0.36$ ,  $p = 0.09$ ) or  $F_{IS}$  (Spearman:  $\rho = 0.21$ ,  $p = 0.32$ ). However, the results of the simulation analysis indicated that correlations between random subsets of two and ten of the microsatellite markers resulted in lower values of  $\rho$  in 17.6% for expected heterozygosity, 54% for observed heterozygosity, 40% for allelic richness and 7% for  $F_{IS}$ . Thus, there is insufficient power to detect correlations between MH and microsatellite loci.

Expected heterozygosity and allelic richness differed significantly for the MH II among management classes (Kruskal Wallis test:  $p = 0.003$ ,  $p = 0.007$ , Figure 2). Introduced populations showed the lowest diversity and native non-stocked populations the highest. This pattern was not evident for microsatellite loci. Expected heterozygosity and allelic richness were significantly higher for MH II genes than for microsatellites in non-stocked native populations (clustered Mann-Whitney-Wilcoxon

test:  $p = 0.002$ ,  $p = 0.008$ ; Figure 2). This was not the case for the other management classes. No significant differences were observed between management classes for observed heterozygosity,  $F_{IS}$  values or effective population size which was inferred from microsatellites (Dawnay et al., 2011). Percentages of molecular variance were 68% within populations, 30% among populations and 2% among individuals for microsatellites and were 51% within populations, 23% among populations and 26% among individuals for the MH II genes.

#### *Population differentiation*

We found a significant correlation between MH II and microsatellite pairwise  $F_{ST}$  estimates (Mantel test DAA:  $P = 0.001$ ,  $r = 0.55$ ; DAB:  $p = 0.02$ ,  $r = 0.38$ ; Figure 3). Pairwise  $F_{ST}$  estimates were not significantly different between MH II and microsatellite estimates across all populations and when comparing non-stocked native and stocked native populations (Figure 4A, B). However, considering only non-stocked native populations pairwise  $F_{ST}$  estimates were significantly lower for MH II genes than for microsatellites (Pairwise Mann-Whitney-Wilcoxon; DAA:  $p = 0.009$ , DAB:  $p = 0.02$ ) (Figure 4C).

Pairwise  $F_{ST}$  estimates significantly greater than zero were found for most population pairs for all genes (Table 2). Dawnay et al. (2011) identified four demographic units based on microsatellites (A-D). Un-rooted neighbour-joining phylogenetic trees suggest a similar pattern of population sub-groups for MH II genes as for neutral markers (Figure 5). However, the Dee population groups with cluster C rather than A

and the Derbyshire Derwent with A rather than D, where they were grouped for neutral markers (Figure 5).

### *Selection*

For the stocked native Aire (AIR) and Dove (DOV) and the introduced Clyde (CLD) populations no evidence for selection was identified by the Ewens-Watterson test for any MH gene and microsatellite results suggested a recent population decline (Supporting Information 1). For the non-stocked native Dee (DEE), Severn (SEV) and Wylfe (WLA/B), as well as for the introduced Eden (EDN) and stocked native Hampshire Avon (HAV) populations allele frequencies deviated significantly from expectations under neutrality for both MH II genes and microsatellites, but in each case the difference between observed and expected allele frequencies was greater for microsatellites, indicating a dominant effect of a recent population decline (Supporting Information 1). Populations that did not show larger significant differences between observed and expected allele frequencies for microsatellites than for MH genes were the non-stocked native Ure (URE) and Wye (WYE) populations. The DAA locus showed evidence for balancing selection for the Ure population and the DAB locus for the Wye population.

### *Simulations*

Simulating the effect of stocking between neutral and MH II markers for different population census sizes, showed that at very low population sizes (census size 500) neutral markers are affected more strongly than MH II markers, as measured by

stronger reductions in observed and expected heterozygosity (Figure 6). There was no significant difference in MH observed heterozygosity between stocked and non-stocked replicates at this population size. In all other cases, there was a significant reduction of MH heterozygosity (observed and expected, Figure 6) after stocking. Comparing the marker types, for higher population sizes (census size 750, 1500, 2000) the effect of stocking was significantly stronger on MH expected heterozygosity than on microsatellites in all cases and for observed heterozygosity for population census sizes of 750 and 1500 (Figure 6). For population sizes of 1500 and 2000 there was no significant effect of stocking on expected heterozygosity on neutral markers and for observed heterozygosity a significant effect of stocking was found only for a population size of 2000 (Figure 6). Looking at the effect of stocking separately for each year, shows that a persisting significant effect on MH heterozygosity is observed at a population size of 750 (Figure 7B).

## Discussion

In order to maintain adaptive genetic variation in threatened populations, it is important to understand how management impacts on functional genetic diversity and evolutionary processes. In this study we compared the performance of neutral and functional markers in informing conservation and management decisions, using salmonids as a model to evaluate the effect of supportive breeding on these different types of genetic markers. Measurements of genetic diversity at functional MH loci could not be predicted by neutral markers. Across different grayling population management classes only non-stocked native populations showed evidence for selection maintaining higher levels of variation at the MH II than at neutral loci. We

implemented a simulation model to test if the removal of natural selection on early life-stages within the hatchery could explain our empirical results. A significant reduction in MH diversity but not neutral diversity was predicted by our model at intermediate population sizes. This is consistent with our empirical results. Further, a significant reduction in the response to selection resulting from supportive breeding was predicted by all simulated scenarios. Overall, our results show clear differences between functional versus neutral genetic loci, confirming the imperative to use adaptive genetic markers to inform conservation decisions (Piertney and Webster, 2008; Sutton et al., 2011). Our results have clear implications for population management involving augmentation, calling into question its efficiency in supporting long term viable populations with high adaptive potential.

We found significant differences in allelic richness and expected heterozygosity for MH II genes, but not microsatellites, across management classes. Although the lowest diversity was found in introduced populations, which might be expected as the consequence of a bottleneck this result is not supported by neutral markers. The loss of diversity in introduced populations was specific to the MH II. Explanations other than founder effects must explain the loss of variation. In a similar study looking at population genetic variation in translocated rainbow trout (*Onchorhynchus mykiss*), Monzón-Argüello et al. (2013) found low MH II diversity relative to neutral markers. These authors attribute this to selection pressures against MH alleles that perhaps did not provide a selective advantage in the novel environment into which they were introduced. Such habitat specific adaptations, where there is a fitness advantage of local genotypes, have been found at the MH II in river and lake populations of three-spined stickleback (Eizaguirre et al., 2012b). Our study further underlines that, using

neutral markers as a surrogate of adaptive genetic variation is unreliable. This observation has been demonstrated in a range of taxa, e.g. mammals (Aguilar et al., 2004), other salmonids (Dionne et al., 2007) and birds (Hartmann et al., 2014). Specific consideration of adaptive markers and likely impacts of demographic history and management on them needs to be a routine part of conservation genomic research.

Few studies (e.g. Schenekar and Weiss, 2017) have focussed on assessing adaptive versus neutral variation specifically in supportive breeding programmes, a practice becoming increasingly adopted as means to effectively manage population declines. Here, significantly higher genetic diversity (allelic richness and expected heterozygosity) of MH genes than microsatellites was observed in non-stocked native populations. Non-stocked native populations also showed significantly lower differentiation for MH II markers than neutral markers. This suggests that balancing selection is acting to retain variation at the MH in natural populations, but that this is not the case for introduced or stocked native populations. On the one hand, stocked native populations with reduced MH II diversity could be undergoing drift and this has removed variation more rapidly at MH II loci due to the combined effect of drift and selection (Ejsmond and Radwan, 2011; Sutton et al., 2011). This seems unlikely because Dawnay et al. (2011) found evidence for bottlenecks in ten populations and these included populations of all three categories (introduced, stocked native and non-stocked native). Thus, there is no evidence that most grayling populations selected for stocking suffered recent and severe population decline, or exhibit lower effective population sizes (Dawnay et al., 2011). Therefore, considering a direct

effect of the stocking process on the efficiency of selection to act within the supplemented population is consistent with our results.

We found no correlation between MH II and neutral markers using any measure of genetic diversity, however we show that for the number of microsatellite markers genotyped (twelve) by Dawnay et al. (2011) and two MH loci, the statistical power for detection is insufficient to identify differences between functional versus neutral loci we analysed. This highlights the importance of caution when making inferences of overall genetic diversity from only a low number of markers (DeWoody and DeWoody, 2005) and the importance of considering power explicitly when designing a programme of sampling. However, we found evidence for recent selection on MH loci as outlined above. Additionally, we found higher within-population amino acid (AA) diversity than between populations, and significantly more even allele frequencies than expected, while we did not observe this for microsatellites.

The levels of diversity reported for the MH II here, compare to those of other salmonids, where generally both the alpha and beta chain show similar levels of diversity (Gómez et al., 2010). This contrasts with other vertebrates ( e.g. chicken, Salomonsen et al., 2003; humans, Reche & Reinherz, 2003), where the alpha chain shows much lower levels of diversity. Two populations, Aire and Severn, significantly departed from HWE due to heterozygote deficiency at the DAB locus (Table 1). Whilst a technical cause, such as allelic drop-out at the DAB locus, cannot be ruled out, elevated (but not significant)  $F_{IS}$  vales have also been found for the same populations at the DAA locus. This is not consistent with uneven reproductive

success between families or a Wahlund effect (Wahlund, 1928) because the pattern is not also shown by neutral loci. To observe higher levels of inbreeding for genes under balancing selection than for neutral markers seems counterintuitive. However, a loss of diversity under the simultaneous effects of selection and drift has been shown both empirically and theoretically (Ejsmond and Radwan, 2011; Sutton et al., 2011). Additionally, MH II mediated mate choice is not necessarily disassortative, seeking highest offspring dissimilarity, but assortative, where particular alleles confer highest resistance, e.g. under frequency dependent selection (Eizaguirre et al., 2009). We report more than 20 times higher proportion of molecular variance found among individuals for the MH II than for microsatellites, which supports an important role of frequency dependent or heterogeneous selection in space and time opposed to overdominant selection. Thus, unbalanced reproductive success for particular MH II genotypes, resulting in elevated inbreeding, might be expected, particularly where competition for mating opportunities is high (Milinski, 2006). This can be for example the case when spawning grounds are scarce (Castric et al., 2002), which has been documented for the Severn population (Lewis, 2006).

Our ability to directly compare native populations before and after stocking is limited by lack of pre- and post-stocking samples. Our simulation model addresses this focussing on plausible genetic effects caused by unequal reproductive contributions of hatchery reared young in relation to naturally produced offspring and the different selective environments experienced by each respectively.



577 The key outcome of our simulation is that the consideration of stocking intensity in  
578 relation to naturally produced offspring is crucial to reduce negative long-term effects  
579 on adaptive genetic diversity. Across taxa, empirical and simulated data need to be  
580 obtained to establish the effects of supplementary breeding on adaptive genetic  
581 variation that underlies fitness. For example, our results show that stocking can have  
582 a strong effect on genetic variation at lower population census sizes (500 and 750)  
583 where the stocking intensity exceeded numbers of naturally produced offspring. The  
584 largest effect of drift was observed at a low census size (500), where the differential  
585 effect of stocking was larger at neutral loci than at MH II loci (Figure 6). With  
586 increasing population census size the effect of drift decreased and in all other cases  
587 variation at the MH II was lost at a higher rate than at neutral markers as a result of  
588 stocking. At a population census size of 750, heterozygosity remained significantly  
589 lower for the MH II in year wise comparisons, but not neutral markers even ten years  
590 after stocking (Figure 7B). Marie et al. (2010) also reported that the loss of genetic  
591 integrity correlates with stocking intensities in brook charr (*Salvelinus fontinalis*).  
592 Even for larger census sizes (1500 and 2000) where the ratio of naturally produced  
593 offspring to stocked offspring was high there was a large effect on MH loci but a  
594 negligible effect on neutral markers. However, the effect was weaker than for smaller  
595 population sizes as would be theoretically predicted. As populations with low census  
596 size would be most likely to be considered for stocking it is important to notice their  
597 vulnerability to genetic deterioration. Furthermore, our results are likely to  
598 underestimate the role of selection, because MH related mate choice was not  
599 considered, though it has been shown to maintain MH diversity in teleost (Consuegra  
600 and Leaniz, 2008; Eizaguirre et al., 2009). Also, we focus specifically on the effect of  
601 the removal of natural selection within the artificial rearing environment and do not

consider adaptation to the hatchery environment, which would be likely to further exacerbate differences in adaptive genetic diversity.

It is interesting from a theoretical point of view that a lower differential effect of stocking on the MH compared to neutral markers was observed at a census size of 500. This could be explained by the expectation that in the population prior to stocking, MH diversity is lost at a greater rate than neutral genetic diversity through the combined forces of drift and selection (Ejsmond and Radwan, 2011; Sutton et al., 2011). Considering that selection becomes less efficient through stocking fish that have not experienced natural selection at the early life stage, the supplemented population is partly alleviated from this additional force, so that the difference between stocked and non-stocked MH diversity is smaller than that observed for neutral markers.

In the simulation model, we assume that a limited number of individuals is selected as brood stock (20 individuals), which reflects current practice. However, selecting a larger number of individuals would likely retain more genetic diversity within the hatchery brood stock and reduce the effect of drift. Also, we assume an initial mortality of stocked fish of 50%, as described in *Salmo trutta* (Pederson, 2003). The survival of stocked grayling has shown to be highly variable, e.g. in some places natural populations do not show any signs of introgression with hatchery stocks, whilst in other places the original population was completely replaced (Persat et al., 2016). Evidence that stocked grayling within UK rivers do survive and contribute to the population is provided by the observation that genetic relationships of stocked

populations agreed with stocking records and through the recapture of stocked individuals (Dawnay et al., 2011). Given the uncertainty around exact rates of initial survival of stocked fish, the most relevant parameters here are the ratios of supplemented individuals relative to the number of offspring naturally produced within the recipient population. This also allows for high transferability of the model predictions to other systems and specific cases.

Whilst the MH represents only a specific locus of adaptive importance, our findings may have implications for loci under selection in a broader sense. Both our empirical results and simulations suggest a dilution effect through the supplementation of a natural population with individuals reared within an artificial environment, which adversely affects the efficiency of selection. Though we evaluated a case of balancing selection, a reduction in the efficiency of selection to act upon a population might be expected to extend to other types of selection. As dynamic adaptive responses are crucial under the pressure of current rates of environmental change conservation management should carefully evaluate the possible inference with natural evolutionary processes. In this context it is important to assess the rate of natural production of a population, which in the case of grayling is frequently restricted by habitat deterioration, which reduces the availability of suitable spawning grounds (Nykänen and Huusko, 2002). This will much better inform the number of individuals to supplement into a natural population and offers the possibility to implement measures of habitat restoration as a first resort where natural reproductive capacities are not fully exploited. This reflects the general need for a more comprehensive evaluation of potential risks and benefits from *ex situ* versus *in*

*situ* management practices before these are implemented in a conservation context (Dolman et al., 2015).

## Conclusions

MH II genes in non-stocked native populations of European grayling showed higher variation than was predicted by microsatellites. We also found significant differences at MH loci between different population management regimes (introduced, stocked native and non-stocked native populations) which were not detected by neutral markers. Our findings highlight the importance of using functional genetic markers to inform the conservation management of genetic diversity (Kirk and Freeland, 2011; Piertney and Webster, 2008). We present evidence consistent with selection maintaining genetic variation in functional loci for non-stocked native populations, which is aligned with results from our simulation model. Simulation results suggest selection is less efficient to maintain genetic variation at functional loci in stocked populations, while the effect is negligible in neutral loci.

Our findings have implications for population conservation management where translocation, reintroduction or population augmentation is practised. Our results highlight the need for a clear understanding of the interaction of selective processes with management actions. Conservation programmes need to more explicitly incorporate and consider possible interference with natural evolutionary and adaptive processes during supplementation, especially considering the current rate of environmental change.

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### *Data Accessibility*

Genotype data is available from DRYAD under doi:10.5061/dryad.dj625ng; raw reads obtained from the Illumina Nanorun are deposited in the NCBI SRA archive under accession number SRP155806;

### *Author contributions*

V.H. performed the MHC genotyping laboratory work, conducted the data analysis and simulations and wrote the manuscript. All authors assisted in the research design and editing of the manuscript. J.S.E. conceived of the project.

### *Supporting Information*

#### *1. Results of the Ewens-Watterson test for MH and microsatellite markers*

Tables:

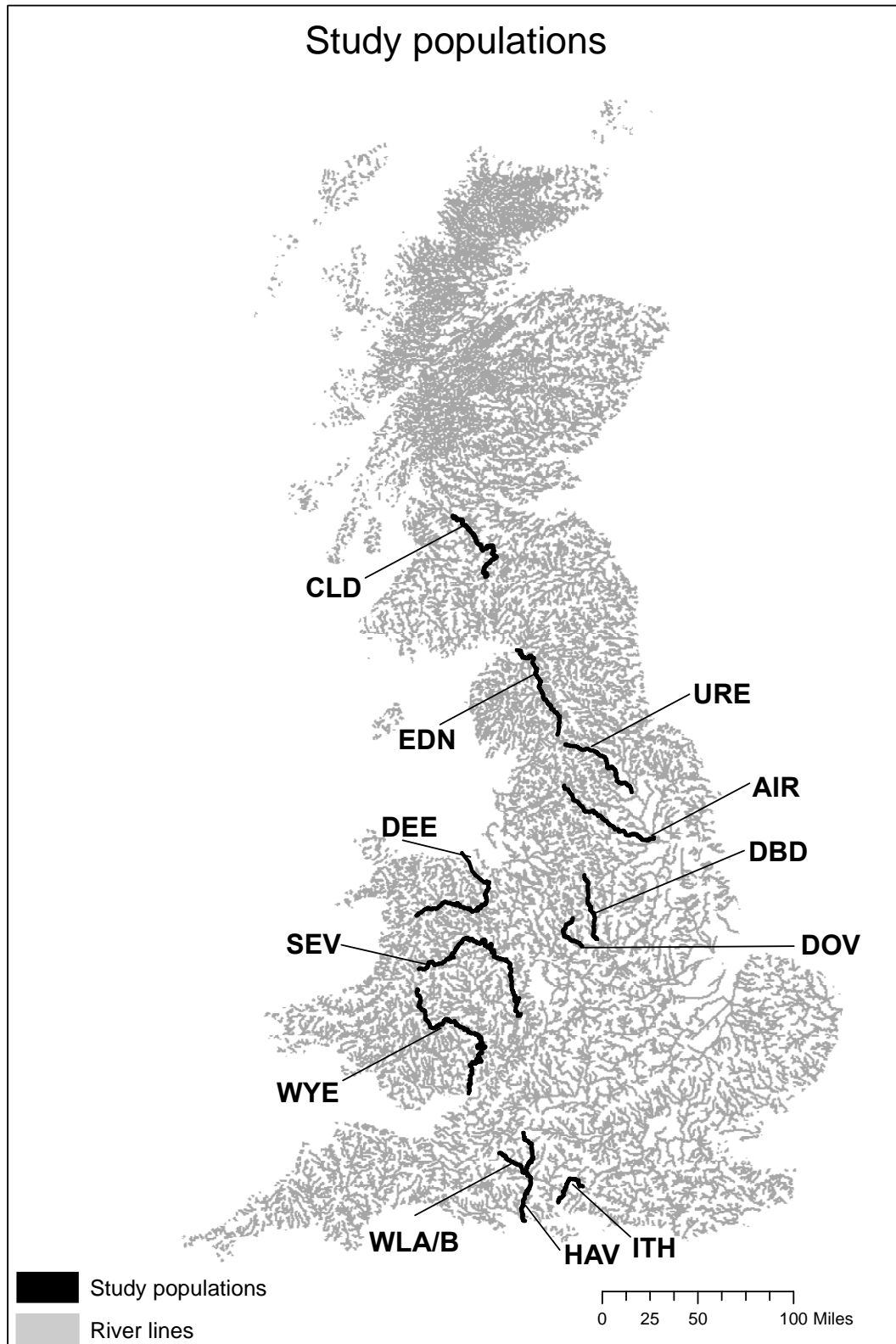
**Table 1:** Sample locations with population classification and summary of genetic diversity for microsatellite markers (from Dawnay et al.(2011)), MH class II  $\alpha$  (DAA) and  $\beta$  chain (DAB). Population classifications are given for non-stocked native (N), stocked native (NS) and introduced (I) populations; number of genotyped samples (N) and allelic richness (Na), expected heterozygosity (He), observed heterozygosity (Ho), inbreeding coefficient ( $F_{IS}$ ), with values showing significant deviation from Hardy-Weinberg equilibrium after Hochberg-Bonferroni correction in bold and number of private alleles (NP) are given. For microsatellite markers estimated effective population size ( $N_e$ ), where (\*) indicates the detection of a bottleneck, is given; for MH loci average amino acid (AA) distance of alleles (PBR) within populations are given and mean pairwise AA distance across individuals for the whole sequence and only for the PBR; population abbreviations are followed as in Dawnay et al (2011)

Microsatellites (from Dawnay et al. (2011))								DAA								DAB							
pop	class	N	Ne	Na	He	Ho	Fis	N	Na	He	Ho	Fis	NP	Mean AA distance alleles PBR	Mean pairwise AA distance all/PBR	N	Na	He	Ho	Fis	NP	Mean AA distance alleles PBR	Mean pairwise AA distance all/PBR
CLD	I	64	68.6*	2.3	0.39	0.37	0.05	40	2	0.31	0.38	-0.22	0	0.44	0.06/0.18	37	2	0.29	0.35	-0.2	0	0.5	0.07/0.18
EDN	I	45	48.7*	2.5	0.4	0.38	0.04	33	3	0.36	0.24	0.33	0	0.29	0.03/0.08	36	3.5	0.34	0.25	0.26	0	0.4	0.04/0.11
ITH	I	50	86.6	2.5	0.39	0.38	0.02	34	2	0.42	0.29	0.31	0	0.25	0.04/0.08	20	2	0.41	0.45	-0.1	0	0.65	0.1/0.29
DEE	N	52	43.2*	3.5	0.54	0.51	0.04	27	6.7	0.8	0.74	0.07	0	0.3	0.08/0.2	26	5.9	0.77	0.54	0.3	0	0.42	0.08/0.21
SEV	N	39	40.8	2.8	0.42	0.41	0.03	31	3	0.53	0.39	0.27	0	0.29	0.04/0.12	30	3.9	0.57	0.23	0.6*	0	0.42	0.03/0.1
URE	N	58	62.5	2.9	0.35	0.32	0.09	31	6	0.8	0.58	0.28	4	0.27	0.08/0.18	30	5	0.59	0.4	0.32	2	0.47	0.08/0.2
WYE	N	55	121	3	0.4	0.4	0	30	3.7	0.64	0.63	0.02	0	0.28	0.06/0.18	22	3	0.65	0.55	0.16	0	0.47	0.08/0.23
WLA/B	N	48/51	33.5*	2.2	0.34	0.32	0.05	34	4	0.73	0.68	0.07	0	0.29	0.08/0.22	25	4	0.75	0.56	0.26	0	0.43	0.09/0.25
AIR	N S	39	63.9	3	0.45	0.44	0.02	31	4	0.59	0.42	0.3	1	0.23	0.05/0.1	29	4	0.54	0.28	0.5*	1	0.42	0.05/0.13
DBD	N S	39	36.9	2.7	0.42	0.45	0.07	35	3.4	0.54	0.57	-0.06	2	0.32	0.1/0.26	36	2	0.5	0.58	-0.18	0	0.5	0.11/0.29
DOV	N S	50	64.4	2.6	0.35	0.32	0.04	26	2.8	0.3	0.23	0.23	0	0.33	0.04/0.1	35	2.9	0.3	0.26	0.14	0	0.51	0.05/0.13
HAV	N S	58	32.5*	2.5	0.42	0.45	0.07	37	4.9	0.73	0.7	0.04	0	0.34	0.09/0.25	33	4.9	0.75	0.73	0.03	0	0.46	0.12/0.33

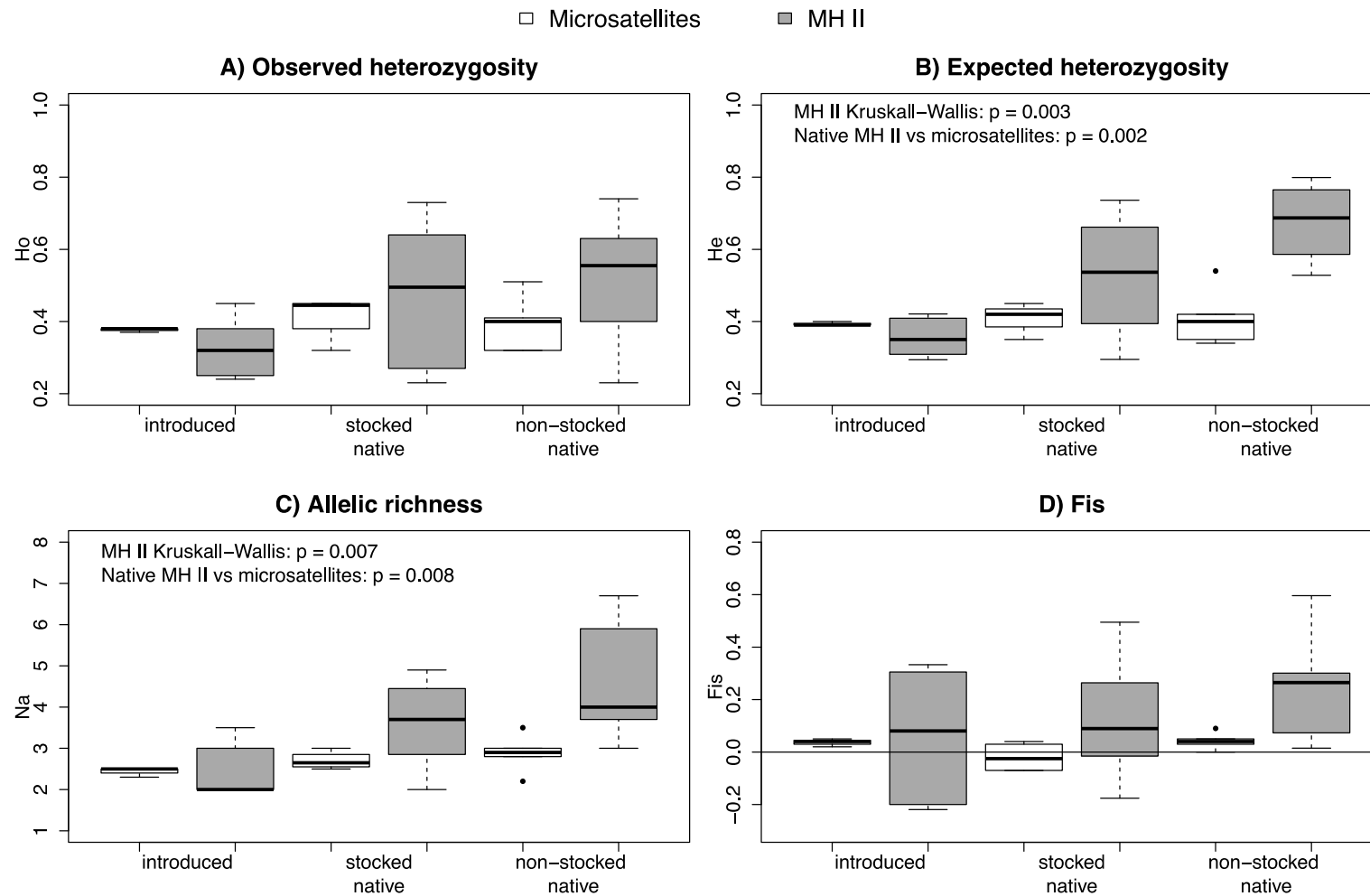
**Table 2:** Pairwise linear  $F_{st}$  ( $F_{st}/(1-F_{st})$ ) between all populations for each gene individually

DAA	AIR	CLD	DEE	DBD	DOV	EDN	HAV	ITH	SEV	URE	WYE
CLD	0.81										
DEE	0.28	0.29									
DBD	0.53	0.14	0.16								
DOV	0.74	-0.01	0.26	0.15							
EDN	0.69	0.01	0.22	0.15	-0.01						
HAV	0.33	0.31	0.05	0.21	0.28	0.25					
ITH	0.65	1.10	0.26	0.74	1.07	0.95	0.33				
SEV	0.73	0.95	0.32	0.28	0.96	0.85	0.48	1.04			
URE	0.28	0.50	0.15	0.31	0.45	0.42	0.19	0.53	0.47		
WYE	0.49	0.52	0.13	0.27	0.49	0.36	0.26	0.71	0.23	0.28	
WLA/B	0.39	0.78	0.10	0.48	0.72	0.66	0.09	0.21	0.57	0.27	0.40
DAB	AIR	CLD	DEE	DBD	DOV	EDN	HAV	ITH	SEV	URE	WYE
CLD	0.99										
DEE	0.45	0.26									
DBD	0.68	0.10	0.14								
DOV	0.66	0.00	0.17	0.07							
EDN	1.03	-0.01	0.27	0.15	0.01						
HAV	0.50	0.37	0.09	0.25	0.26	0.39					
ITH	0.69	0.87	0.27	0.53	0.59	0.91	0.29				
SEV	0.89	0.98	0.26	0.37	0.74	1.04	0.50	0.71			
URE	0.43	0.12	0.13	0.15	0.06	0.12	0.19	0.39	0.64		
WYE	0.72	0.76	0.13	0.37	0.55	0.76	0.37	0.54	0.09	0.43	
WLA/B	0.52	0.63	0.07	0.39	0.43	0.67	0.01	0.28	0.52	0.28	0.38

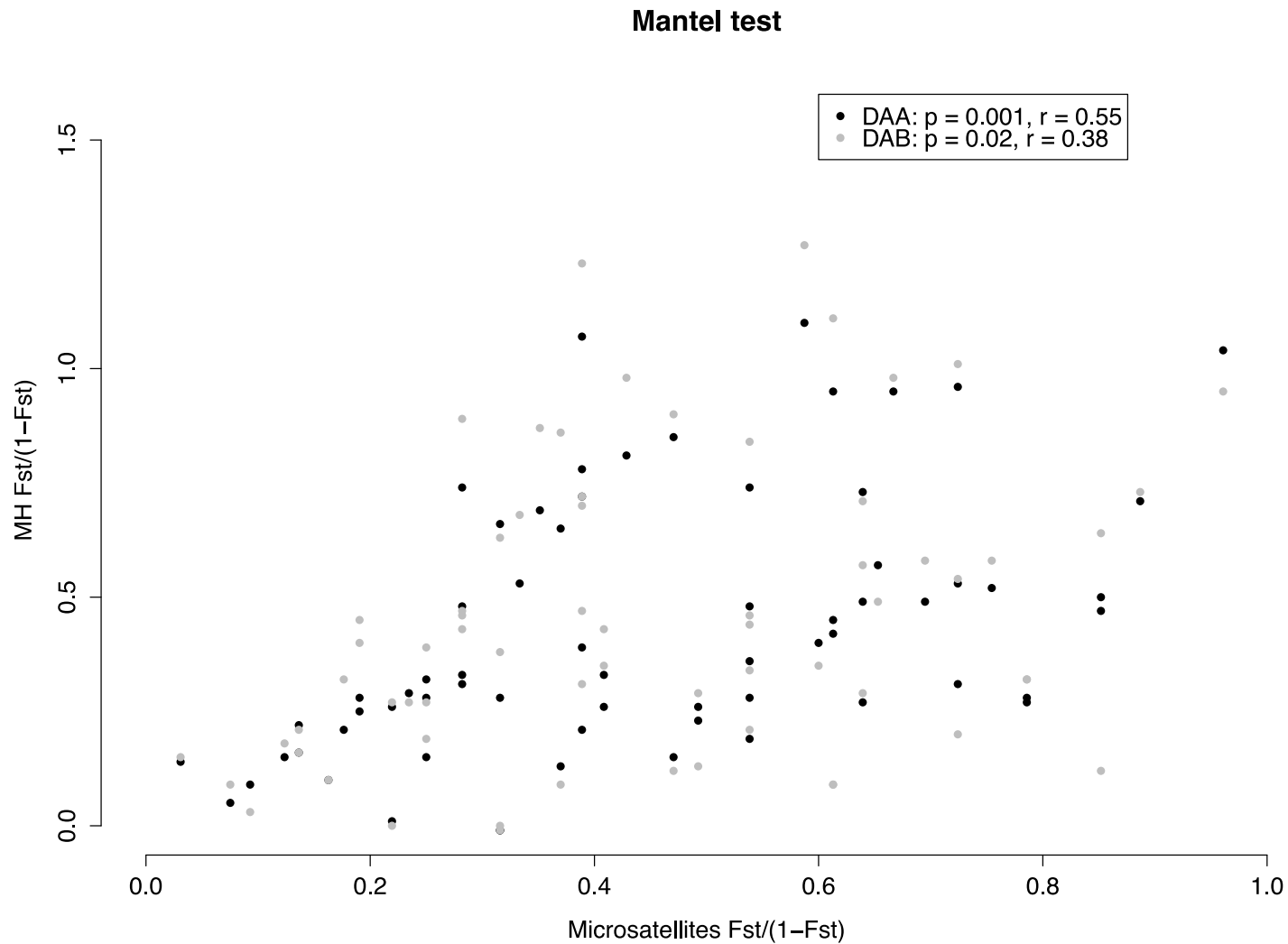
Figures



**Figure 1:** Populations genotyped at MH II DAA and DAB markers in this study in black: Clyde (CLD), Dee (DEE), Derbyshire Derwent (DBD), Dove (DOV), Eden (EDN), Hampshire Avon (HAV), Itchen (ITH), Severn (SEV), Ure (URE), Wye (WYE), Wylfe (WLA/B); River lines across the UK are shown in grey

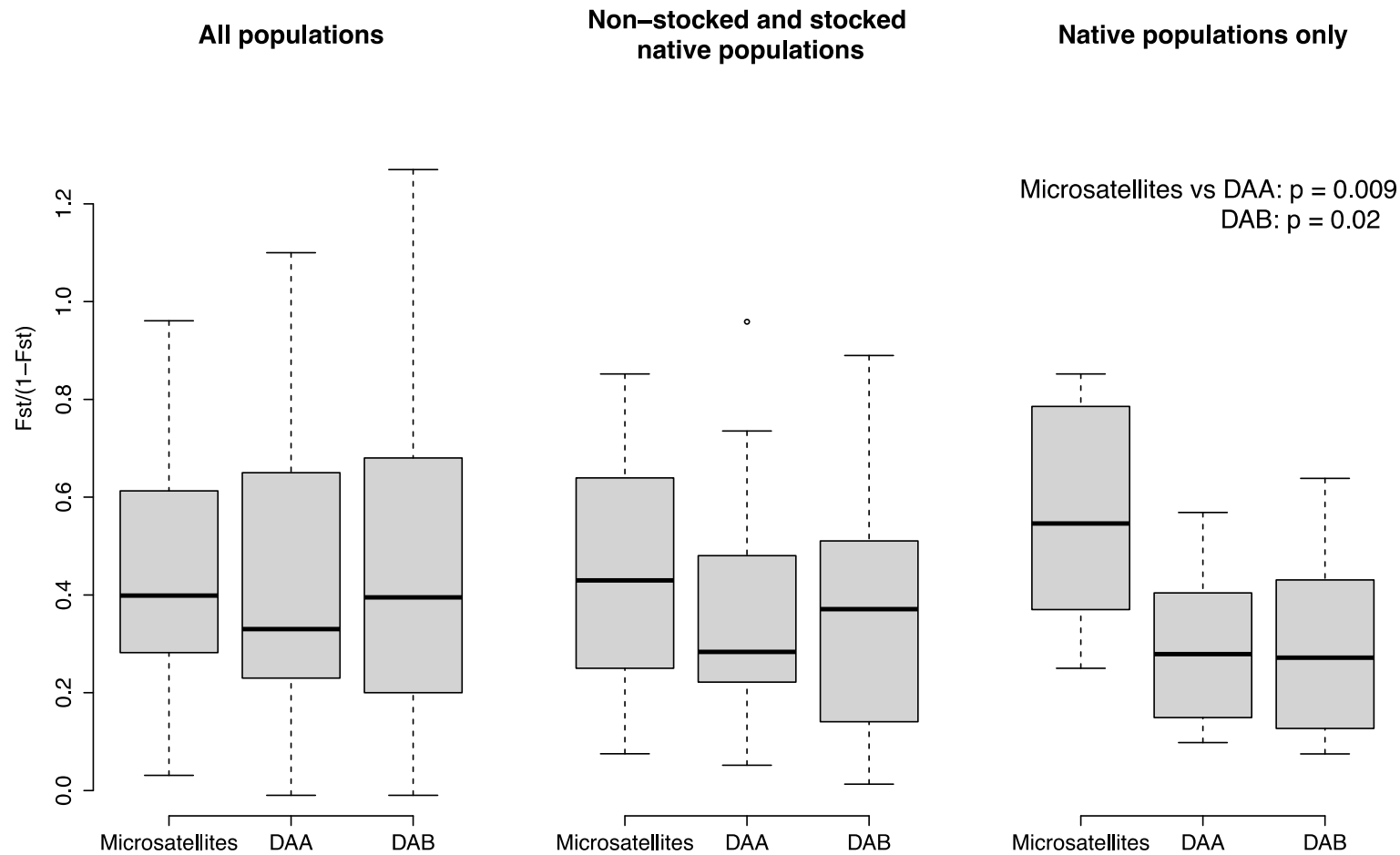


**Figure 2:** Comparison between microsatellite and MH II measurements of genetic diversity (A: observed heterozygosity, B: expected heterozygosity, C: allelic richness ( $N_a$ ) and D: inbreeding coefficient ( $F_{is}$ )) across management classes consisting of introduced, stocked native and non-stocked native populations; Significant differences after correction for multiple testing are shown for Kruskal-Wallis tests across management classes and clustered Mann-Whitney-Wilcoxon test between markers within each management class;



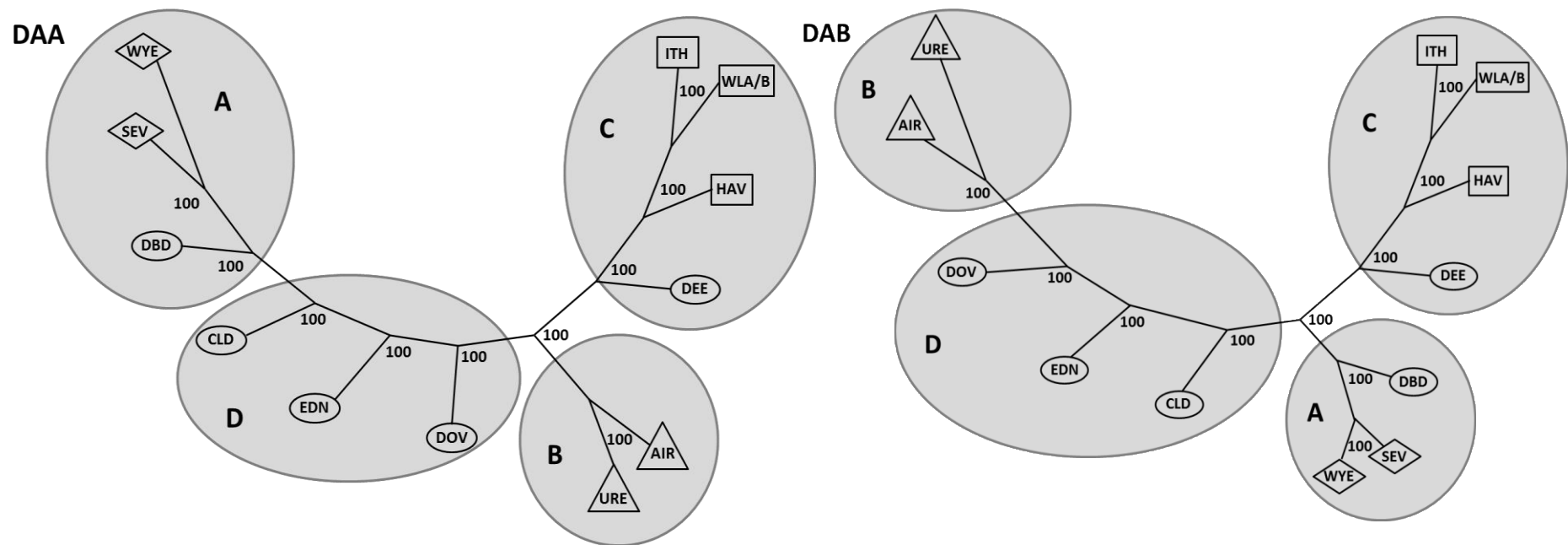
**Figure 3:** Relationship of pairwise  $F_{ST}/(1-F_{ST})$  of microsatellites and MH II DAA and DAB genes:  $P$  values are given with Spearman correlation coefficients in significant cases;

## Population divergence for different markers

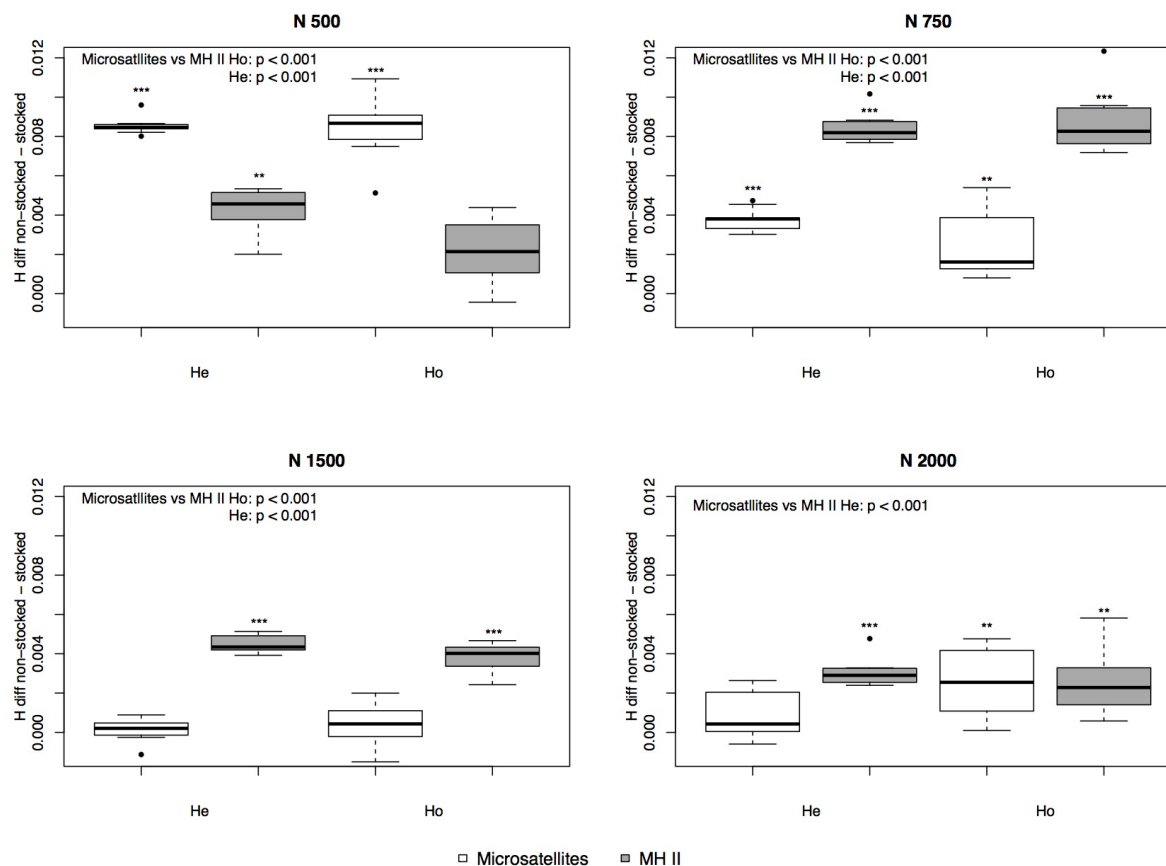


**Figure 4:** Population divergence measured as pairwise  $F_{ST}/(1 - F_{ST})$  for the different markers studied: P values of Mann-Whitney-Wilcoxon tests with significant differences between markers are given; A: between all populations; B: considering only native and native stocked populations; C: considering only purely native populations;





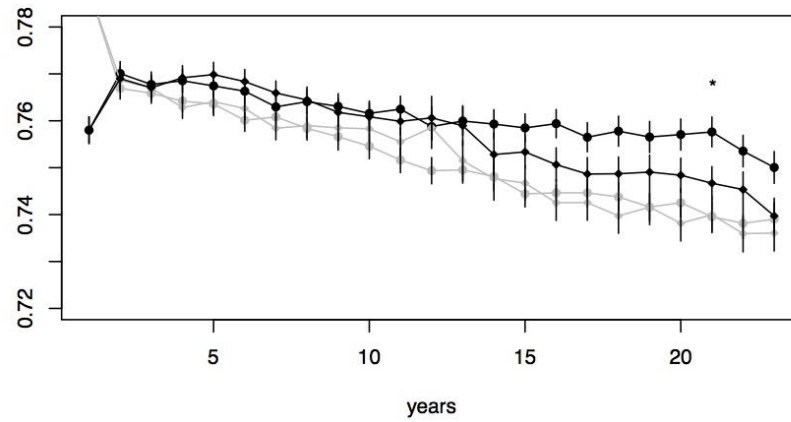
**Figure 5:** Unrooted phylogenetic trees based on Nei's genetic distance for DAA and DAB of the MH class II: Bootstrap support based on 2000 replicates is given; shapes around population abbreviations reflect the assignment to clusters based on neutral markers in Dawnay et al. (2011), with diamonds for cluster A, triangles for cluster B, boxes for cluster C and circles for cluster D;



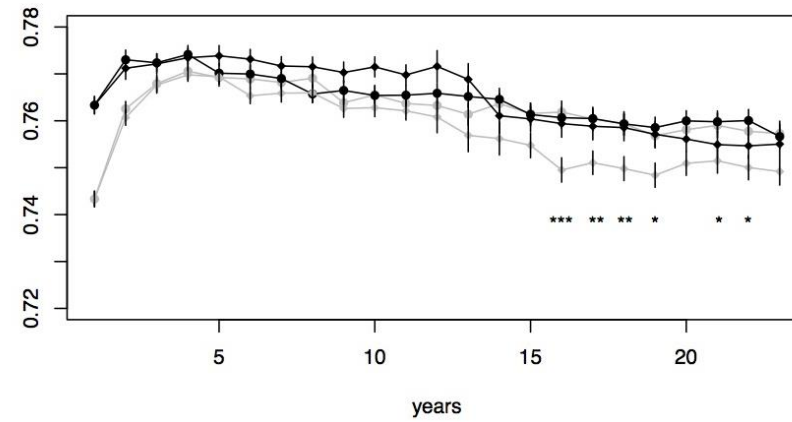
**Figure 6:** Difference in expected ( $H_e$ ) and observed heterozygosity ( $H_o$ ) between non-stocked and stocked simulated replicates (averaged across the ten following years after stocking events) are shown for neutral and MH markers at population census sizes of 500, 750, 1500 and 2000 (the ratio of naturally produced offspring to those stocked were roughly 0.5:1, 0.8:1, 1.6:1 and 2:1 respectively); Significant differences after correction for multiple testing are shown above plots with \*\* indicating  $p$ -values below 0.01 and \*\*\*  $p$ -values below 0.001; significant comparison between neutral and MH marker differences are printed;

# A) observed heterozygosity over time in stocked and non-stocked replicates

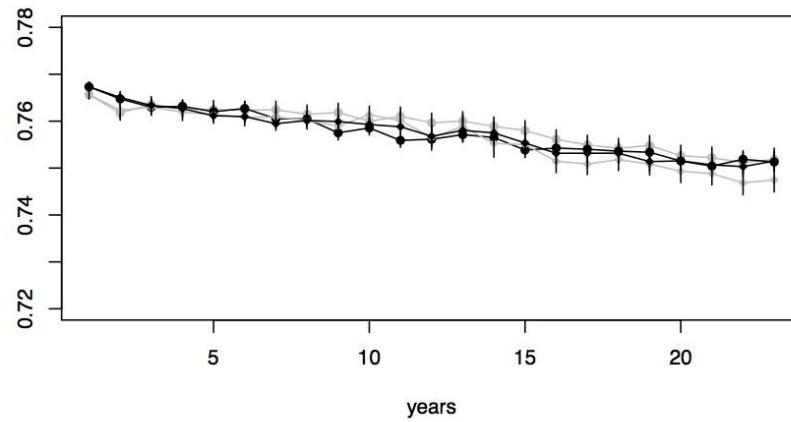
N 500



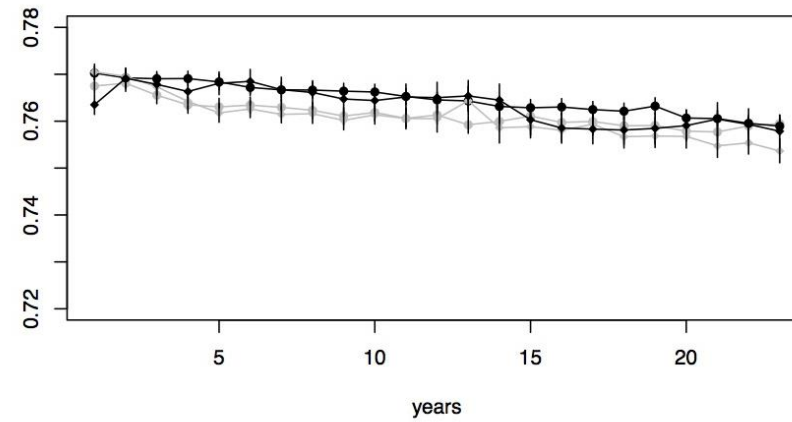
N 750



N 1500



N 2000



● Microsatellites non-stocked  
◆ MH II stocked

◆ Microsatellites stocked  
● MH II non-stocked

**B) expected heterozygosity over time in stocked and non-stocked replicates**

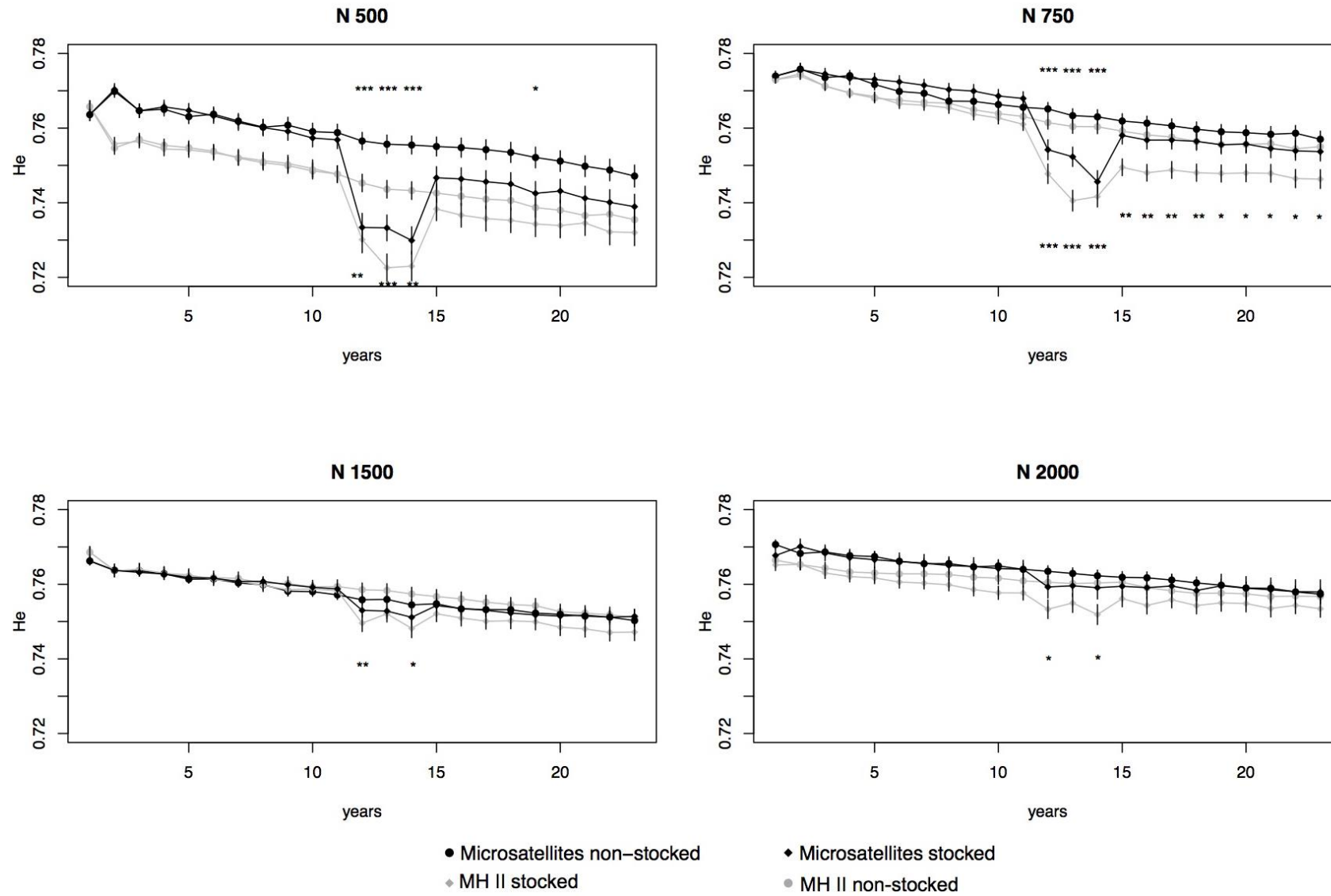


Figure 7: Measurements of observed (A) and expected heterozygosity (B) are averaged across two MH II and two neutral loci and 100 replicates respectively; results are shown when stocking was implemented at year 11, 12 and 13 or when no stocking was implemented. Significant differences after correcting for multiple testing (Hochberg and Benjamini, 1990) between stocked and non-stocked replicates are indicated (Mann-Whitney Wilcoxon tests): \*\*\*  $p < 0.001$ , \*\*  $p < 0.01$ , \*  $p < 0.05$